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(54) Title: HYBRID REAGENTS CAPABLE OF SELECTIVELY RELEASING MOLECULES INTO CELLS

(57) Abstract

Hybrid reagents comprising a first portion having an affinity for a cellular target and a second portion having an affinity for a bioactive molecule are described, said hybrid reagents being capable of selectively releasing the bioactive molecule in response to a change in pH. The hybrid reagents of the present invention can be used diagnostically or therapeutically.

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HYBRID REAGENTS CAPABLE OF SELECTIVELY RELEASING MOLECULES INTO CELLS

Background of the Invention

Hybrid antibodies are antibodies or aggregates of antibodies which are specific for two different antigens. Hybrid antibodies can comprise a single antibody or fragment having a bispecific antigen binding region (two different variable regions) or aggregates of two or more antibodies of different specificities.

Different methods of preparing hybrid antibodies have been reported. Auditore-Hargreaves teaches processes for preparing hybrid antibodies by generating "half molecules" from two parent antibodies and subsequently associating different half molecules. See U.S. Patents 4,470,925 (1984) and 4,479,895 (1984). Using this process, various hybrid antibodies were prepared with specificities for horseradish peroxidase, glucose oxidase and theophylline.

Reading describes production of antibodies having binding specificities for two desired antigens using a quadroma cell or a trioma cell. See U.S. Patent 4,474,893 (1984). The quadroma cell is the fusion product of two different hybridoma cells, each of which produce an antibody with a different specificity. A trioma cell is the fusion product of a hybridoma and a lymphocyte which produces antibodies with two different binding specificities.

Segal et al. describe target specific cross-linked heteroantibodies which are used as cytotoxic agents in U.S. Patent 4,676,980 (1987). Staerz et al. (1986), PNAS, 83:1453-1457, teach the use of a hybrid antibody that can focus effective T cell activity and Milstein et al. (1983), Nature, 305:537-539, describe the use of hybrid antibodies in immunohistochemistry.

Raso et al., Cancer Research, 41:2073-2078 (1981) disclose the use of hybrid antibodies with dual specificity for the plant toxin, ricin, and immunoglobulin-bearing target cells. The hybrid antibodies were constructed in vitro and the attachment of the hybrid antibody-ricin complex to the human target cells was observed using fluorescein labeled antibodies. Upon binding, the human target cells were selectively killed by the hybrid-delivered toxin.

Prior to the use of hybrid antibodies, chemical crosslinking or nonspecific absorption methods were used to couple drugs and/or toxins to antibody carriers. These agents possess certain limitations due to the nature of the linkage. The linkage may alter the drug or toxin such that the therapeutic or toxic activity is reduced. Moreover, cleavage of the covalent bond may be rate-limiting for the action of toxin inside the cell.

The use of hybrid antibodies obviated some of the problems encountered with chemical crosslinking or non-specific absorption methods; however, new problems were created. Because the drug or toxin is

bound to an antibody, the therapeutic or toxic activity is generally inhibited. Hybrid antibody-delivered toxins or drugs are inactive when bound to the antibody and only become active upon release. However, the hybrid antibodies currently available have no mechanism for releasing the toxin or drug from the respective antibody binding region when the hybrid antibody reaches the target site or the interior of the cell. Instead, they rely on fortuitous dissociation. As a result, relatively large quantities of hybrid antibodies containing drugs or toxins must be administered, because only a small amount of the drug or toxin will dissociate and become active.

Summary of the Invention

This invention pertains to hybrid reagents comprising a first portion having an affinity for a cellular target (e.g., antibody, virus, ligand, receptor or molecule) and a second portion having an affinity for a bioactive molecule (e.g., a toxin, drug, enzyme or metal). The hybrid reagents can be administered *in vivo* where they bind to the external surface of a cell. Once bound to the cell, receptor-mediated endocytosis serves to pinch off the surface of the cell forming an endosome, which has a lower pH than either outside or within the rest of the cell. In response to the pH change inside the endosome, the hybrid reagents of the present invention selectively

release the bioactive molecule. Once released, the bioactive molecule is free to perform its function.

Therefore, a major advantage of hybrid reagents of this invention over currently available hybrid antibodies, which rely on fortuitous dissociation of bioactive molecules, is that less of the hybrid and bioactive molecule need to be administered to produce the desired diagnostic or therapeutic effect.

The present invention also encompasses pharmaceutical compositions comprising said hybrid reagents having a bioactive molecule bound thereto, methods of immunotherapy and a method for selecting antibodies or fragments thereof capable of binding a bioactive molecule at one pH and releasing that molecule in response to a change in pH.

Brief Description of the Figures

Figure 1 is a schematic diagram depicting the delivery to a cell of a bioactive molecule from a hybrid reagent by receptor mediated endocytosis and release of the bioactive molecule in response to the lower pH found within a cellular endosome.

Figure 2 is a graph plotting the percent dissociation (i.e., release) of monoclonal antibody 6B3 from diptheria toxin over 100 minutes time at a pH of 4.5 and temperatures of 22°C and 37°C.

Figure 3 is a graph plotting the percent dissociation of monoclonal antibody 6B3 from diptheria toxin over 30 minutes time at pH 5.0 and pH 4.5 at 37°C.

Figure 4 is a graph plotting the percent dissociation of monoclonal antibodies 5A7 and 1F3 from diphtheria toxin over 60 minutes time at pH 5.0 at 37°C.

Figure 5 is a graph plotting the percent incorporation of ^{3}H leucine over 180 minutes time as a measure of protein synthesis inactivation by native diphtheria toxin and hybrid-delivered CRM107 in H-meso cells.

Figure 6 is a graph plotting the toxicity dose-response curve for hybrids and conjugates incubated for 16 hrs. with transferrin receptor positive CEM cells.

Figure 7 is a graph plotting the toxicity dose-response curve for HIV and transferrin receptor directed hybrids on HIV-infected 8E5 cells.

Detailed Description of the Invention

The hybrid reagents of this invention comprise a first portion having an affinity for a cellular target and a second portion having an affinity for a bioactive molecule (e.g., a toxin, drug, metal or an enzyme). The hybrid reagents can be administered in vivo where they bind to the external surface of a cell. Once bound to the cell, receptor-mediated endocytosis serves to pinch off the surface of the cell forming an endosome, which has a lower pH than either outside or within the rest of the cell. In response to the change in pH within the endosome,

the hybrid reagents selectively release the bioactive molecule. The first portion of the hybrid can be, for example, a ligand (e.g., transport proteins such as transferrin, interleukin-2, LDL), a growth factor (e.g., EGF, PDGF), an antibody, a hormone, a receptor molecule (e.g., recombinant CD4), a virus, or a fragment thereof and the second portion is an antibody or an antibody fragment.

The first portion of the hybrid reagent has an affinity for a cellular target, such as an antigenic or receptor site on the surface or inside a cell (i.e., a cell surface antigen or cell surface receptor). Examples of cellular targets are Ig, common acute lymphoblastic leukemia antigen (CALLA), B1, gp26, Ia, transferrin receptor, EBV transformation antigen and the receptors for ligands such as interleukin-2, MSH, insulin, thyroglobulin, LHRH and NGF. Viral proteins on the surface of infected cells (e.g., HIV-infected T-lymphocyte) can also serve as targets for antibody and receptor guided hybrid reagents.

The second portion of the hybrid reagent is an antibody or antibody fragment that has an affinity for a bioactive molecule at one pH and releases the bioactive molecule in response to a change in pH. This bonding and release may be due to a number of mechanisms. For example, the second portion of the hybrid reagent may have an affinity for a bioactive molecule that undergoes a conformational change in response to a change in pH. Such molecules can be identified by using physical or other methods known

in the art (e.g., circular dichroism, fluorescence). As another example, the second portion of the hybrid reagent may ionically bond to a bioactive molecule at one pH and the ionic bond may break in response to a change in pH.

A method for isolating antibodies that dissociate from molecules in response to a change in pH is described in detail in Example 1. In general, antibodies against a bioactive molecule are prepared using known techniques. Clone supernatants are then assayed for the ability to bind the molecule at the first selected pH. Clones testing positive for binding ability are screened to isolate those that release the molecule at a second selected pH. For example, antibodies that bind a bioactive molecule at physiologic pH (pH about 6.5 to 7.5) can be tested to isolate those clones that release the molecules at acidic pH (pH less than 6.5).

Examples of bioactive molecules are plant or bacterial toxins, drugs, enzymes and metals. Examples of useful toxins are diphtheria toxin, pseudomonas exotoxin, ricin, pokeweed antiviral peptide (PAP), and tricathecum. The toxins can also be genetically or chemically altered or mutated such as CRM107 (Laird J. Virol., 19:220-227 (1976)) and HA48DT and HA51DT (Myers et al., J. Biol. Chem., 263:17122-17127 (1988)). Drugs which can be used in the invention are for example, interferon, insulin, and methotrexate. Examples of metals which can be used in the invention are radiometals (e.g., Tc-99m, In-111, Cu-67, Pd-109, Pd-103, Re-188,

Au-198, Au-199, Ru-97, Hg-197, Ag-111, Bi-212, Os-191 and Pb-203) and non-radioactive metals (e.g., zinc).

Figure 1 illustrates receptor-mediated endocytosis of a hybrid reagent-molecule complex. The first portion of the hybrid reagent binds to the external surface of the cell, which becomes pinched off to form an endosome. Endosomes have a pH lower than (e.g., pH about 4.5-5.5) the pH either outside or within the rest of the cell (e.g., pH about 6.5-7.5) (Geisow, M.L. and W.H. Evans, Exp. Cell Res., 150:36-46 (1984)). Therefore, by using a hybrid reagent in which the first portion has an affinity for a cell surface component and the second portion has an affinity for a bioactive molecule at physiologic pH and dissociates from the bioactive molecule in response to acidic pH, a molecule can be delivered into a cell and released within acidic compartments of cells, such as cell endosomes.

The hybrid reagents can be produced by joining together the first and second portions using known techniques (e.g., chemical coupling, cell fusion, or genetic engineering techniques). The hybrid reagents are preferably made by chemically coupling the two portions together. For example, a disulfide linkage using N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) as the crosslinking agent can be used (Raso et al., NATO Advanced Studies Institute, 82:119-138 (1984)). Both portions become sparingly substituted with pyridyldisulfide groups which are reduced to thiols on one of the portions. Upon

mixing of the two portions, the free thiols on one of the portions readily reacts with the unreduced groups on the second portion and form disulfide linkages. The resulting hybrids can then be purified using gel filtration.

When the first and second portions of the hybrid reagent are both antibodies, two whole parental antibodies may be joined together to produce the hybrid reagent (i.e., hybrid antibody). A variety of crosslinking agents, such as protein A, carbodiimide, and N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) can be used to link the whole parental antibodies (Kranz et al., Proc. Natl. Acad. Sci. USA, 78:587 (1981); U.S. Patent 4,474,893)).

The hybrid antibodies can also be produced by chemically joining parental antibody fragments containing a sufficient portion of the antigen binding region to allow the fragment to bind to its respective antigen (Nisonoff et al., Arch. Biochem. Biophys., 93:460-467 (1961) and Raso et al., Cancer Research, 41:2073-2078 (1981)). The two types of parental antibodies (i.e., one type will become the first portion of the hybrid antibody and the other type will become the second portion) can then be separately digested with pepsin. Bivalent $F(ab')_2$ molecules are obtained after a separation step such as chromatography. Equal amounts of the $2F(ab')_2$ types can then be mixed and after reducing their inter-heavy chain disulfide linkages, the resulting Fab' fragments are allowed to randomly reassemble into $F(ab')_2$ dimers with dual specificity. The dual

specificities of the hybrid product can be verified using cell-based and solid phase assays which use radioactive or fluorescent probes (Raso, V., Immunol. Reviews, 62:93-117 (1982)).

Alternatively, the intrinsic disulfide links of the $F(ab')_2$ molecules can be reduced to thiols and the vicinal thiols generated can be stabilized (e.g., with sodium arsenite). Ellman's reagent can be used to activate the vicinal thiols on one type of the Fab' fragments. Upon mixture of the reduced Fab' fragment with an activated Fab' fragment, an exclusively bi-specific hybrid will be formed (Brennan, M., et al., Science, 228:81-83 (1985)).

The hybrid antibodies can also be produced using cell fusion techniques as described in U.S. Patent 4,474,893, to Reading. In this technique, hybridoma cells which secrete the parental antibodies are fused together to form quadroma or trioma cells. These quadroma and trioma cells secrete bi-specific antibodies possessing the antigen binding regions of both parental antibodies.

In addition, the hybrid antibodies can be produced using genetic engineering techniques. In these procedures, DNA encoding the heavy and light chain variable regions of each of the parental antibodies are introduced into an appropriate host cell, preferably a lymphoid cell (e.g., a myeloma cell). The transformed cell can then synthesize, assemble and secrete the hybrid antibody.

The parental antibodies used to produce the hybrid antibody can be selected from those presently

available or can be specially prepared. The parental antibodies can be obtained using conventional monoclonal antibody methodology, (e.g., the standard somatic cell hybridization techniques of Kohler and Milstein, Nature, 256:495 (1975)).

Suitable antibodies which are specific towards tumor associated antigens and are therefore appropriate to comprise the first portion of the hybrid reagent, are for example, 7D3, directed against the human transferrin receptor, (Griffin et al., Cancer Res., 47:4266 (1987)); C19, directed against the carcinoembryonic antigen, (Griffin et al., J. Biol Resp. Modif., 1:194 (1982)); 260F9, directed against a breast cancer antigen, (Bjorn et al., Cancer Res., 45:1214 (1985)); 96.5 directed against a melanoma associated antigen, (Casellas et al., In. J. Cancer, 30:437 (1982)); 45-2D9, directed against an oncogene product, (Roth et al., J. Immunol., 136:2305 (1986)) and J-5, directed against the common acute lyphoblastic leukemia antigen, (Raso et al., Cancer Res., 42:457 (1982)).

Suitable antibodies which are specific towards diptheria toxin and are capable of releasing the toxin in response to a change in pH from physiologic to acidic, are D5E8, D1F3, D3E1, D6B3, D5D5, D1D5, D5F5 and D4B7. These antibodies are therefore appropriate to comprise the second portion of the hybrid reagent.

The hybrid reagents described herein can be used diagnostically. For example, hybrid molecules comprising a first portion which has an affinity for

a tumor cell and a second portion which has an affinity for a radiometal can be used to deposit radiometal within tumor cells and thereby obtain a scintographic image of the tumor.

Hybrid reagents of this invention can also be used therapeutically. For example, hybrid molecules comprising a first portion having an affinity for a viral-associated antigen (e.g., an HIV antigen) or a viral-associated receptor and a second portion having an affinity for a bioactive molecule, can be used therapeutically to kill or otherwise modify virus infected cells. Similarly, hybrid molecules comprising a first portion having an affinity for a tumor-associated antigen or a tumor-associated receptor and a second portion having an affinity for a bioactive molecule can be used therapeutically to kill or otherwise modify tumor cells.

When the hybrid reagent described herein is used in a pharmaceutical composition, it can be administered by a wide variety of techniques. For example, intravenously, parenterally, transdermally subcutaneously or via an implanted reservoir containing the hybrid molecule. The form in which the hybrid molecule will be administered (e.g., solution, emulsion) will depend on the route by which it is administered. The quantity of the hybrid molecule to be administered will be determined on an individual basis and will be based at least in part on consideration of the individual's size, the severity of the symptoms to be treated, and the result sought.

This invention is further illustrated by the following examples.

Example 1 The Isolation of Anti-Diphtheria Toxin Antibodies Capable of Releasing a Molecule at a Selected pH

Mice were immunized with progressively increasing doses of active diphtheria toxin (1 μg - 3 μg I.P.) or a high dose of formalin-inactivated diphtheria toxoid (100 μg I.P.). Following a booster injection of the immunogen, spleens were removed and fused with NS-1 cells to generate hybridomas (Kohler and Milstein, Nature, 256:495 (1975)). Supernatants from microtiter wells with clones were assayed for the ability to bind ^{125}I-diphtheria toxin using a polyethylene glycol precipitation method. Antibody positive supernatants usually bound 25,000 cpm while negatives and controls bound only 4,000 cpm. In a typical fusion approximately 35 positive clones were obtained from the spleen of a single animal.

A second assay was developed in order to examine the influence of pH on the interaction between diphtheria toxin and the different monoclonal antibodies. Diphtheria toxin (100 μl at 300 μg/ml) was absorbed to polyvinyl microtiter wells, excell was washed off with PBS. Antibody (100 μl at 1-50 μg/ml) was then added, allowed to react for two hours and the plate was washed with PBS. Attached antibody was revealed by subsequent addition of a ^{125}I-goat antimouse IgG reagent (background was

approximately 100 cpm, positive clones bound approximately 1,000-3,000 cpm).

To test for pH effects on toxin release, the antibody was allowed to bind to the immobilized diphtheria toxin for two hours in replicate wells and then a small volume of concentrated buffer was added to provide a final pH of 7.0, 5.0 or 4.5. Dissociation was allowed to proceed for different time intervals (5-90 minutes) at either 23°C or 37°C (normal body temperature). Released antibody was quickly washed off the plates with PBS and the amount remaining was quantified using a ^{125}I-goat antimouse IgG probe. This method was used to identify 23 clones producing antibody which rapidly dissociated from diphtheria toxin at a pH of 4.5 and eight clones having antibody that was sensitive to release at a pH of 5.0. No release occurred at a pH of 7.0.

The time-course of dissociation at pH 4.5 for one of these monoclonal antibodies (D6B3) is shown in Figure 2. At 23°C the rate of release was slower and less complete than at 37°C. Approximately 80 percent of the antibody initially bound dissociated from diphtheria toxin and most of this occurred within the first 5 minutes. It is known that the diphtheria toxin remains attached to the assay plate since binding of monoclonal antibodies derived from different clones remained completely unaffected by the same acid conditions.

Figure 3 shows that the binding interaction of this D6B3 antibody was much less sensitive to

release at pH 5.0, with only 25 percent having dissociated by 30 minutes in contrast to 80 percent at pH 4.5 The kinetics of release for two monoclonal antibodies which did dissociate at pH 5.0, at 37°C is shown in Figure 4. The binding interaction between D5A7 and diphtheria toxin was even disrupted at pH levels as high as 5.5. Thus a substantial fraction of diphtheria toxin was rapidly relinquished by these different antibodies at the precise pH and temperature conditions found in endosomal vesicles and other acidic compartments within cells (Geisow, J.L. and W.H. Evans, Exp. Cell Res., 150:36-46 (1984)).

The pH-dependent break-up of antibody and toxin was shown to be based upon conformational changes in the toxin. Thus, the $t_{1/2}$≈1-2 min for the acid triggered dissociation of antibody and toxin is close to the $t_{1/2}$=30 sec for the pH-induced transition of free toxin (Blewitt, M.G., et al., Biochem., 24:5458-5464 (1985)). Moreover, the D6B3 antibody bound to formalin stabilized diphtheria toxoid at pH 7.0 but did not release when the pH was reduced to pH 4.5. Apparently the chemical crosslinking of toxoid prevented the pH-induced transition which allows D6B3 to dissociate from native toxin.

Example 2 Hybrid-Mediated Delivery of ^{125}I-Diphtheria Toxin to Cells

Hybrid antibodies were formed with various anti-diphtheria toxin antibodies by linking them to

anti-transferrin receptor monoclonal antibodies by a method previously described (Raso, F., et al., NATO Advanced Studies Institute, 82:119-138 (1984)). The dual specificity and cell targeting capability of these hybrids was demonstrated using ^{125}I-diphtheria toxin (hereinafter ^{125}I-DT). CEM cells derived from a patient with T-cell leukemia (Foley, G.E., et al., Cancer, 18:522-529 (1965)), which have abundant transferrin receptor on their surface, were used as a test line for anti-transferrin receptor/ anti-diphtheria toxin hybrids and two different routes of delivery were tested. The cells were either pre-treated with the hybrid and washed so that the empty toxin binding sites of surface-bound hybrids could then capture subsequently added ^{125}I-DT; or hybrid plus ^{125}I-DT were pre-complexed and then used as a single agent for reaction with the cell surface transferrin receptors.

CEM cells were incubated with the components designated in Table I for 30 minutes at 0° and then washed with PBS to remove unbound hybrid. They were then exposed to ^{125}I-DT for 30 minutes at 0°, washed with PBS and counted to measure the amount bound to cells.

The results in Table I show that cells exposed to an anti-transferrin receptor/anti-diphtheria toxin hybrid (7D3/D1F3) bound five times higher levels of ^{125}I-DT than untreated cells. This enhanced binding was receptor-specific since pre-occupying the target epitope using excess unmodified 7D3 antibody blocked hybrid attachment and

subsequent ^{125}I-DT binding (Table I). Hybrids formed with different anti-diphtheria toxin monoclonal antibodies (D4B7 and D5E8) showed similar toxin binding properties (Table I).

TABLE I

Binding of ^{125}I-DT to Hybrid-Coated CEM Cells

Pretreatment	CPM Bound
None	888
7D3/D1F3 Hybrid	4,381
Excess 7D3 plus 7D3/D1F3 Hybrid	973
None	556
7D3/D4B7 Hybrid	4,306
7D3/D5E8 Hybrid	5,657

CEM cells were treated for 1 hour at 0°C with an equivalent amount of ^{125}I-DT either alone in PBS or pre-complexed at 22° for 15′ to hybrid at 10^{-8}M (Table II). Following treatment, the cells were washed with PBS and counted. Table II shows that significant delivery over the basal binding levels was attained even though the concentration of complex used to treat these cells was relatively low (10^{-8}M).

TABLE II

Delivery of Hybrid-Complexed ^{125}I-DT to CEM Cells

Treatment	CPM Bound
^{125}I-DT alone	1,649
7D3/D4B7 Hybrid - ^{125}I-DT complex	13,116
7D3/D5E8 Hybrid - ^{125}I-DT complex	15,297

Example 3 Plate Assay for Dual Specificity of HIV-Directed Hybrids

An anti-HIV monoclonal antibody was elicited using a synthetic envelope protein and used to form the HIV-specific hybrid (anti-HIV/D5E8) by coupling it to an anti-diphtheria toxin antibody (D5E8) following a method previously described (Raso, F., et al., NATO Advanced Studies Institute, 82:119-138 (1984)). A solid-phase radioimmunoassay was devised by adsorbing the envelope peptide antigen to the wells of polyvinyl microtitre plates. PBS and either antibody or hybrid at $6x10^{-9}$M was then added to the well for 2 hrs, and any unbound reagent was washed off using PBS. The dual specificity of the hybrid was demonstrated after allowing it to bind to the coated plate via its HIV-specific combining sites and then revealing its presence by binding ^{125}I-CRM107 to the free toxin-specific sites of the composite molecule. Table III shows that the anti-HIV/D5E8 hybrid bound ^{125}I-CRM107 while anti-HIV alone bound no toxin even though it was

attached to the plate as evidenced by using an ^{125}I-goat anti-mouse IgG probe.

TABLE III

Plate Assay to Demonstrate the Binding of Anti-HIV Antibody and Hybrid

	Amount Bound (CPM)	
	^{125}I-CRM107	^{125}I-G/M
PBS	307	253
anti-HIV	112	1,501
anti-HIV/D5E8 Hybrid	1,245	

Example 4 Hybrid-Mediated Cytotoxicity of a Mutated Form of Diphtheria Toxin

The availability of genetically or chemically altered diphtheria toxin cogeners (e.g., CRM107) with no capacity for attaching to cells provides an added dimension to the hybrid delivery approach. The cell-binding defect which makes these analogs non-toxic to cells can be restored via the hybrid carrier moiety so that its lethal action is aimed exclusively at the selected cell surface target.

Human mesothelioma cells (H-Meso) were used to test the effectiveness of anti-transferrin receptor/anti-diphtheria toxin hybrids (7D3/D1F3 and 7D3/D5E8) for restoring the full cytotoxic potential of CRM107. The H-meso cells were incubated for 2 hours at 37°C with 4 x 10^{-8}M CRM107 alone;

(4×10^{-8}M) CRM107 in combination with the hybrids 7D3/D5E8 or 7D3/D1F3 at 1×10^{-8}M, or 4×10^{-8}M CRM107 in combination with the hybrids (1×10^{-8}M) plus excess anti-receptor antibody (7D3) (10^{-5}M). Cells were then pulse labeled with ^{3}H-leucine for 30 min. H-meso cells in media to which 10mM NH_4Cl was added were also incubated with the same components.

The data in Table IV show that while CRM107 alone was incapable of entering cells and inhibiting protein synthesis, it became a very potent and rapid-acting cytotoxin when used in combination with the hybrid antibodies. This lethal action was dependent upon hybrid-mediated delivery to transferrin receptors since little toxicity was obtained when these sites were blocked by including an excess of free anti-receptor antibody (7D3) during the 2 hour incubation time (Table IV).

The acid environment of intracellular compartments is essential for cytotoxicity since this induces the release of CRM107 from the antibody and translocation into the cytosol where it inactivates elongation factor 2. This condition was demonstrated by adding NH_4Cl to the cells. This weak base, which is known to raise vesicle pH, greatly reduced the ability of the hybrid-CRM107 combination to kill H-Meso cells (Table IV). The same experiments were carried out using the anti-HIV/D5E8 hybrid (2×10^{-8}M) plus CRM107 (4×10^{-8}M) using HIV-infected 8E5 cells as the target (Folks, T.M., et al., J. Exp. Med., 164:280-290 (1986)). The same acid-dependency was demonstrated (Table V).

TABLE IV

Hybrid-Mediated Cytotoxicity of CRM107 Tested on Human Mesothelioma Cells (2-hr Assay); Transferrin Receptor Specificity and Acid-Dependency

	^{3}H-Leucine Incorporation (CPM)	Inhibition (Percent)
H-Meso Cells	92,560	--
+CRM107	90,755	2
+7D3/D5E8 + CRM107	1,605	98
+7D3/D1F3 + CRM107	8,050	91
+excess 7D3 + 7D3/D5E8 + CRM107	52,325	43
+excess 7D3 + 7D3/D1F3 + CRM107	53,960	42
H-Meso Cells + 10 mM NH_4Cl	92,435	--
+CRM107	76,885	17
+7D3/D5E8 + CRM107	52,105	44
+7D3/D1F3 + CRM107	80,802	13

TABLE V

Acid-Dependency of Hybrid-Mediated Cytotoxicity of CRM107 on HIV-Positive 8E5 Cells

	^{3}H-Leucine Incorporation (CPM)	Inhibition (percent)
HIV-Positive + 8E5 cells alone	103,955	-
+CRM107	85,140	18
+NH_4Cl + CRM107	82,115	21
+anti-HIV/D5E8 + CRM107	21,820	79
+NH_4Cl + anti-HIV/D5E8 + CRM107	78,985	24

The transferrin receptor directed hybrid-CRM107 complex was assayed on human colon adenocarcinoma cells to determine if the same high cytotoxic potency found for the H-Meso and HIV-infected 8E5 cell lines extended to alternative malignant cell types. The combined action of CRM107 plus hybrid at 10^{-8}M produced extensive cell kill within two hours and its potency was comparable to 10^{-7}M native diphtheria toxin (Table VI). These results indicate that hybrid-delivery not only renders CRM107 cytotoxic to cells but also suggests that its entry via the transferrin pathway is as efficient as diphtheria toxin uptake by its usual mechanism. Moreover, a transferrin/ D5E8 conjugate was constructed to examine if transferrin itself would

mediate delivery of CRM107 into cells. In fact, this natural ligand coupled to the anti-diphtheria toxin monoclonal antibody (D5E8) provided a similar level of toxicity as the anti-transferrin receptor (7D3) guided hybrid.

TABLE VI

Lethal Effects of Anti-Transferrin Receptor Directed Hybrid Plus CRM107 on Human Colon Adenocarcinoma Cells (2-hr. Assay)

	^{3}H-Leucine Incorporation (CPM)	Inhibition (Percent)
LS174T cells	54,070	--
+CRM107 (10^{-7}M)	48,355	11
+7D3/D5E8 (10^{-8}M) + CRM107 (10^{-7}M)	1,930	96
+Diphtheria Toxin (10^{-7}M)	1,785	97
+Diphtheria Toxin (10^{-8}M)	6,295	88

In addition to using the transferrin receptor as a target for hybrid delivery, the common acute lymphoblastic leukemia antigen (CALLA) was similarly tested as a site of entry into CALLA-bearing Nalm-1 leukemia cells (Raso, V., et al., Cancer Res., 42:457-464 (1982)). An anti-CALLA/D5E8 hybrid was formed and examined for its ability to kill these cells in combination with CRM107 following the protocol set forth for H-meso cells and anti-transferrin receptor/anti-diphtheria toxin. However,

incubation was carried out for 6 hours at the same temperature (Table VII).

Good cell kill was achieved by targeting the hybrid-CRM107 to this distinct membrane site; however, the longer incubation time required suggests that entry and/or release of toxin was slower than for transferrin receptor directed agents.

TABLE VII

CALLA-Directed Cytotoxic Action of Hybrid-CRM107 on Nalm 1 Cells (6-hr. Assay)

	^{3}H-Leucine Incorporated (CPM)	Inhibition (Percent)
Nalm-1 Cells	22,130	--
+ anti-CALLA/D5E8	24,110	0
+ CRM107	22,820	0
+ anti-CALLA/D5E8 + CRM107	4,080	82

Example 5 Kinetics of Cytotoxicity in H-Meso Cells

One of the fundamental premises underlying the acid-triggered hybrid carrier concept predicts that this mode of delivery will not interfere with the normal mechanism of toxin action after specific targeting has been achieved. A critical measure of toxin efficiency can be obtained by monitoring the kinetics of inhibition of protein synthesis. This parameter accurately indicates how rapidly toxin gains access to its target in the cytosol (e.g.,

elongation factor 2) and was therefore used to evaluate hybrid-delivered CRM107 (Figure 5).

H-Meso cells were incubated at 37°C for the designated intervals with either 10^{-8}M diphtheria toxin, 10^{-8}M CRM107, or the anti-transferrin receptor/anti-diphtheria toxin hybrid (7D3/D3E1)-CRM107 combination at 10^{-8}M. The cells were then pulse labeled with ^{3}H-leucine for 30 minutes to measure the extent incorporation into protein compared to untreated control cells. The time course of protein synthesis inhibition as reflected by ^{3}H-leucine incorporation, for H-Meso cells incubated with 10^{-8}M diptheria toxin alone, 10^{-8}M CRM107 alone or with the anti-transferrin receptor/anti-diphtheria toxin hybrid (7D3/D3E1) plus CRM107 at 10^{-8}M was then measured.

Figure 5 shows that both native toxin and the CRM107 hybrid combination gave identical kinetics profiles which were characterized by a 30-40 minute lag period followed by a rapid inactivation phase with $t_{1/2}$ = 24 minutes and $t_{1/2}$ = 26 minutes respectively. Unbound CRM107 alone at 10^{-8}M had no effect on the ability of the cells to synthesize protein. The fact that hybrid-delivered CRM107 killed cells as fast as native diphtheria toxin suggests that its release from the antibody combining site was unimpeded and that there was no interruption of the normal course of events required for its lethal action.

Finally, a covalently-coupled anti-transferrin receptor-CRM107 conjugate (7D3-CRM107) was

constructed by standard disulfide-linkage methods and its cytotoxic effect compared with the effect produced by the 7D3/D5E8 hybrid plus CRM107. Transferrin receptor positive CEM cells were incubated for 16 hours at 37°C with the designated concentrations of the 7D3/D5E8 hybrid plus 10^{-7}M CRM107, the 7D3-CRM107 disulfide-linked covalent conjugate and native diphtheria toxin, CRM107 alone or 7D3/D5E8 hybrid alone. The cells were then pulse labeled with ^{3}H-leucine for 30 minutes and the amount of incorporation into protein was compared with untreated control cells.

Figure 6 shows the toxicity dose response curves of the hybrid, the conjugate and native diphtheria toxin. The conjugate, 7D3-CRM107 was cytotoxic to transferrin receptor positive cells, the kinetics of cell killing was much slower than that found for hybrid-delivered CRM107. CEM cells are not particularly sensitive to diphtheria toxin as reflected in the $ID_{50} = 2 \times 10^{-9}$M obtained with native toxin. The transferrin-receptor directed 7D3-CRM107 conjugate was slightly more effective, giving an $ID_{50} = 1 \times 10^{-9}$M. In contrast, hybrid-delivered CRM107 ($ID_{50} = 4 \times 10^{-12}$ M) was 250-fold more potent than the covalent conjugate, based upon the concentration of hybrid added. Neither the 7D3/D5E8 hybrid alone nor CRM107 alone had an effect upon the cells. These results indicate that covalent coupling can impede toxin action since the disulfide-linked 7D3-CRM107 conjugate was slower

acting and less potent than the corresponding 7D3/D5E8 hybrid delivered CRM107.

Figure 7 shows dose response curves for inhibition of protein synthesis in HIV-infected 8E5 cells after 16 hr exposure to CRM107 plus hybrids directed against either HIV or transferrin receptors on the cell membrane. The ID_{50} for the anti-HIV/D5E8 hybrid plus CRM107 was $2x10^{-9}M$ but this reagent became 10-times more potent when nicked CRM107 (cleaved at a specific site using trypsin) was used (ID_{50} = $2x10^{-10}M$). It is believed that proteolytic cleavage is a prerequisite for toxic activity and normally occurs at the cell surface or in subcellular compartments. This anti-HIV hybrid-mediated cytotoxicity was blocked by neutralizing intracellular compartments with NH_4Cl (Table V) and the uninfected control cell line was not affected by hybrid-delivered CRM107.

Equivalents

Those skilled in the art will know, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention as described herein. These and all other equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A hybrid reagent comprising a first portion having an affinity for a cellular target and a second portion having an affinity for a bioactive molecule, said hybrid reagent being capable of selectively releasing the bioactive molecule in response to a change in pH.

2. A hybrid reagent according to Claim 1, wherein the first portion is selected from the group consisting of ligands, growth factors, cell receptors, antibodies, transport proteins, hormones and viruses or fragments thereof and the second portion is an antibody or antibody fragment.

3. A hybrid reagent according to Claim 2, wherein the change in pH is from physiologic to acidic.

4. A hybrid reagent according to Claim 3, wherein the bioactive molecule is a toxin, an enzyme, a drug or a metal.

5. A hybrid reagent according to Claim 4, wherein the first portion is the anti-transferrin receptor monoclonal antibody 7D3 or fragments thereof and the second portion is a monoclonal antibody specific towards diptheria toxin selected from the group consisting of D5E8, D1F3, D3E1, D6B3, D5D5, D1D5, D5F5 and D4B7.

6. A pharmaceutical composition comprising, a hybrid reagent having an affinity for a cellular target and having a bioactive molecule bound thereto, said hybrid reagent being capable of selectively releasing the bioactive molecule in response to a change in pH.

7. The pharmaceutical composition of Claim 6, wherein the change in pH is from physiologic to acidic.

8. A pharmaceutical composition comprising, a hybrid reagent having an affinity for a cellular target and having a bioactive molecule bound thereto, said hybrid reagent being capable of selectively releasing the bioactive molecule into an endosome within a cell.

9. A method of immunotherapy, comprising: administering to a subject a hybrid reagent having an affinity for a cell surface antigen and having a bioactive molecule capable of killing or otherwise modifying the cell bound thereto, said hybrid reagent being capable of selectively releasing the bioactive molecule in response to a change in pH.

10. The method of Claim 9, wherein the change in pH is from physiologic to acidic.

11. The method of Claim 10 wherein the cell surface antigen is a tumor associated antigen.

12. The method of Claim 10 wherein the cell surface antigen is a viral-associated antigen.

13. The method of Claim 12 wherein the viral-associated antigen is from Human Immunodeficiency Virus (HIV).

14. A method of immunotherapy comprising administering to a subject a hybrid reagent having an affinity for a cell surface receptor and having a bioactive molecule capable of killing or otherwise modifying the cell bound thereto, said hybrid reagent being capable of selectively releasing the bioactive molecule in response to a change in pH.

15. The method of Claim 14 wherein the change in pH is from physiologic to acidic.

16. The method of Claim 15 wherein the cell surface receptor is a tumor-associated receptor.

17. The method of claim 15 wherein the cell surface receptor is a viral-associated receptor.

18. A hybrid antibody having a first specificity for a cell surface antigen and a second specificity for a bioactive molecule, said

hybrid antibody being capable of selectively releasing the bioactive molecule in response to a change in pH.

19. A hybrid antibody of Claim 18, wherein the change in pH is from physiologic to acidic.

20. A process for selecting an antibody that binds an antigen at a first selected pH and releases said antigen at a second selected pH, comprising:

a) providing immobilized antigen in dilute buffer at a first selected pH and contacting said antigen with antibodies;

b) allowing the antibodies and immobilized antigen to bind;

c) selecting the antibodies that bind to the antigen and adding to said antibodies a small volume of concentrated buffer to provide a second selected pH;

d) selecting antibodies that release bound antigen at the second pH.

21. An antibody or fragment thereof capable of binding an antigen at a first selected pH and releasing said antigen at a second selected pH.

22. The antibody of Claim 21 selected from the group consisting of D5E8, D1F3, D3E1, D6B3, D5F5, D1D5, D5D5 and D4B7.

23. A method of obtaining a scintographic image of a tumor in a subject, comprising:

introducing into the subject a hybrid reagent comprising a first portion having an affinity for a tumor associated antigen and a second portion having an affinity for a radiometal, said hybrid reagent being capable of binding a radiometal at a first selected pH and releasing the radiometal at a second selected pH;

allowing the hybrid molecule to localize at the tumor; and

scanning the subject with a gamma camera to obtain an image of the tumor.

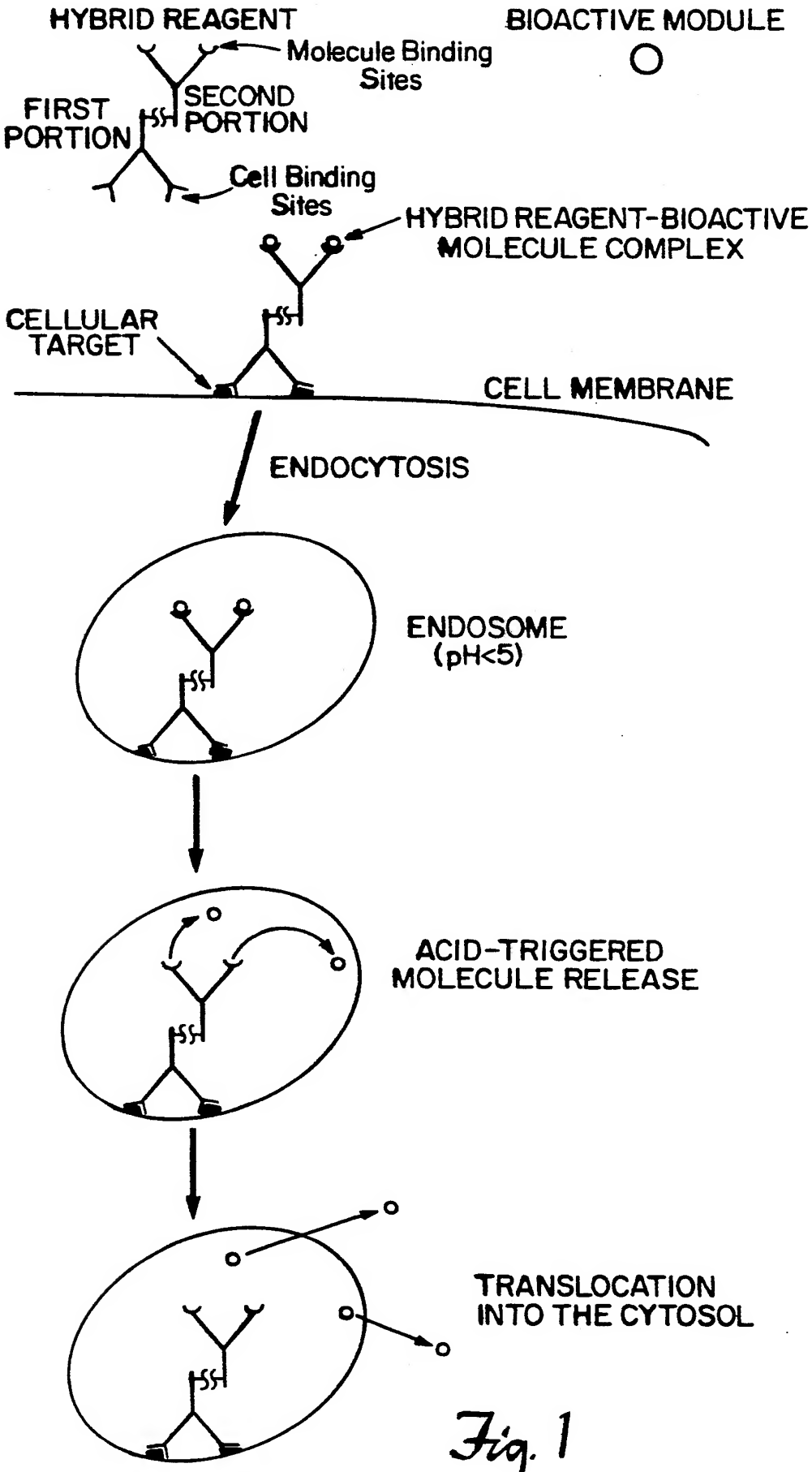
HYBRID REAGENT
BIOACTIVE MODULE
Molecule Binding Sites
SECOND PORTION
FIRST PORTION
Cell Binding Sites
HYBRID REAGENT-BIOACTIVE MOLECULE COMPLEX
CELLULAR TARGET
CELL MEMBRANE
ENDOCYTOSIS
ENDOSOME (pH<5)
ACID-TRIGGERED MOLECULE RELEASE
TRANSLOCATION INTO THE CYTOSOL

Fig. 1

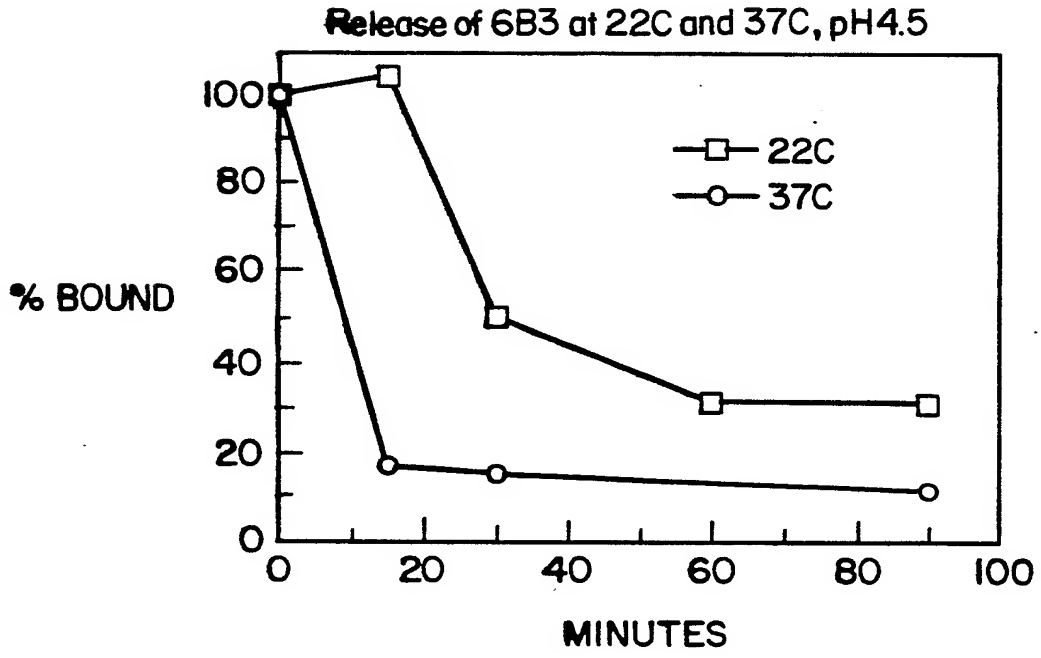
Release of 6B3 at 22C and 37C, pH4.5
% BOUND
100
80
60
40
20
0
0
20
40
60
80
100
MINUTES
22C
37C

Fig. 2

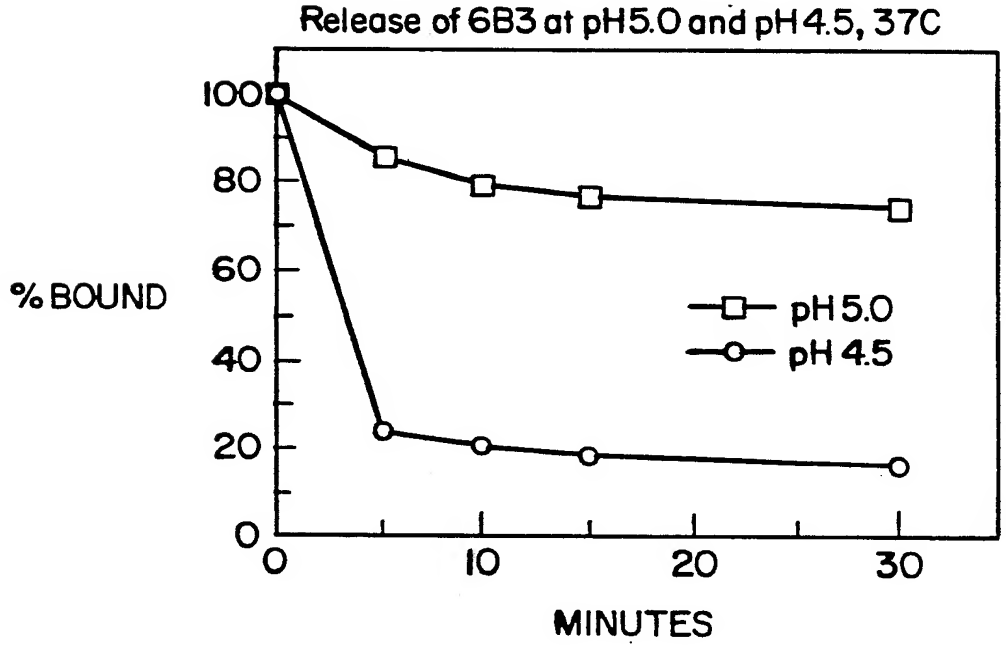
Release of 6B3 at pH5.0 and pH4.5, 37C
% BOUND
100
80
60
40
20
0
0
10
20
30
MINUTES
pH 5.0
pH 4.5

Fig. 3

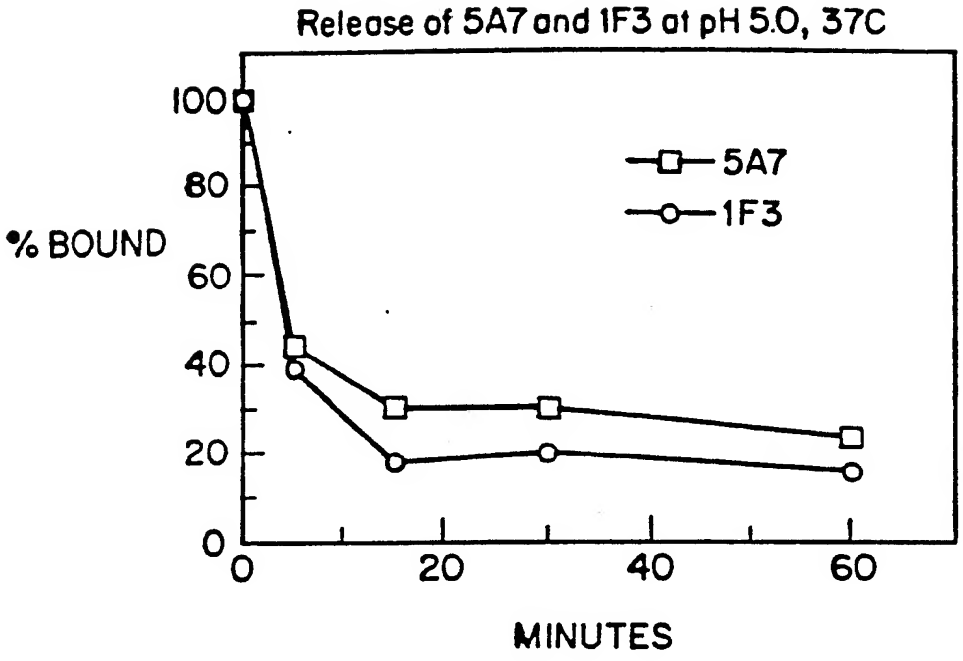
Release of 5A7 and 1F3 at pH 5.0, 37C
% BOUND
100
80
60
40
20
0
0
20
40
60
MINUTES
5A7
1F3

Fig. 4

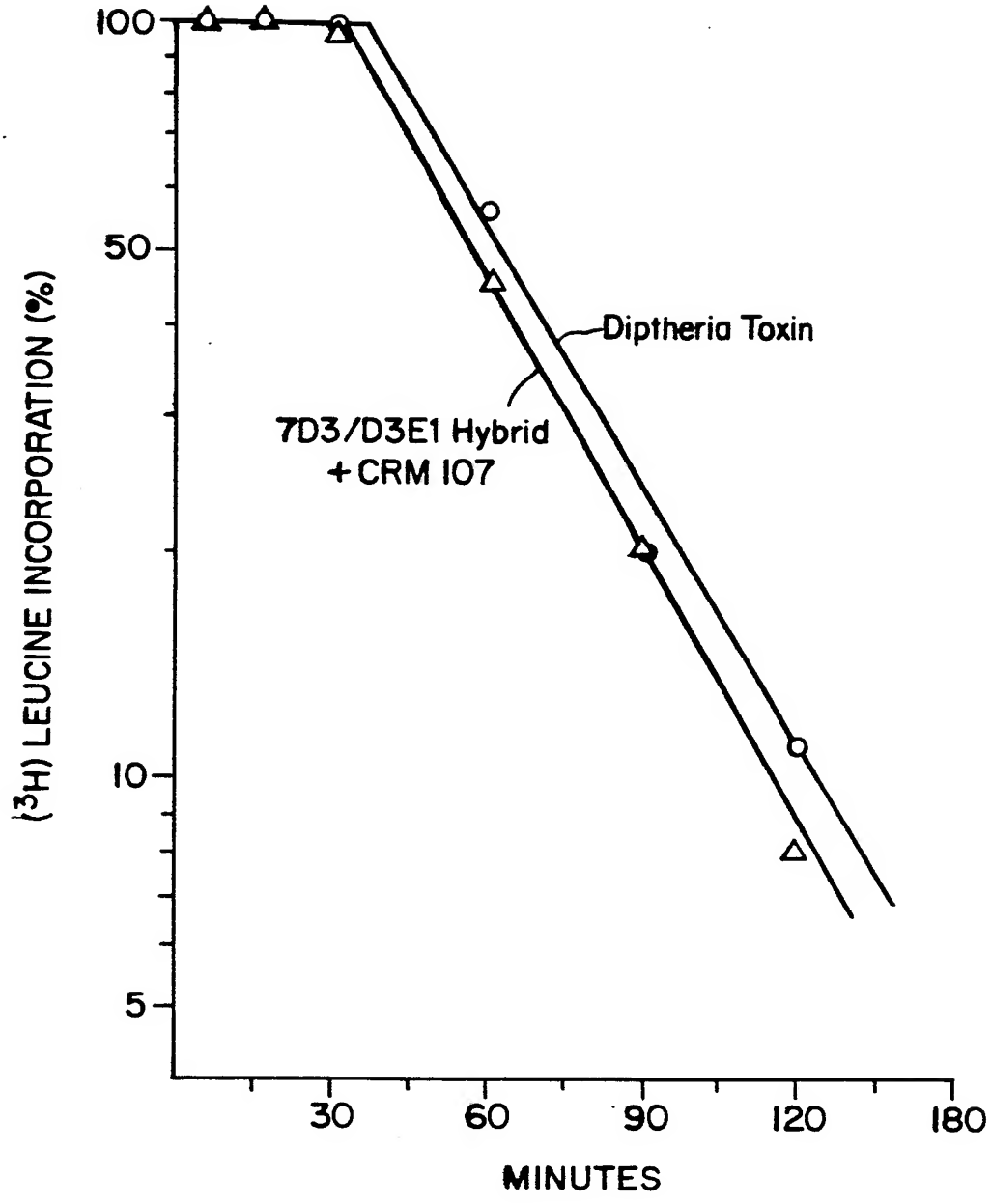
100
50
10
5
(3H) LEUCINE INCORPORATION (%)
Diptheria Toxin
7D3/D3E1 Hybrid
+ CRM 107
30
60
90
120
180
MINUTES

Fig. 5

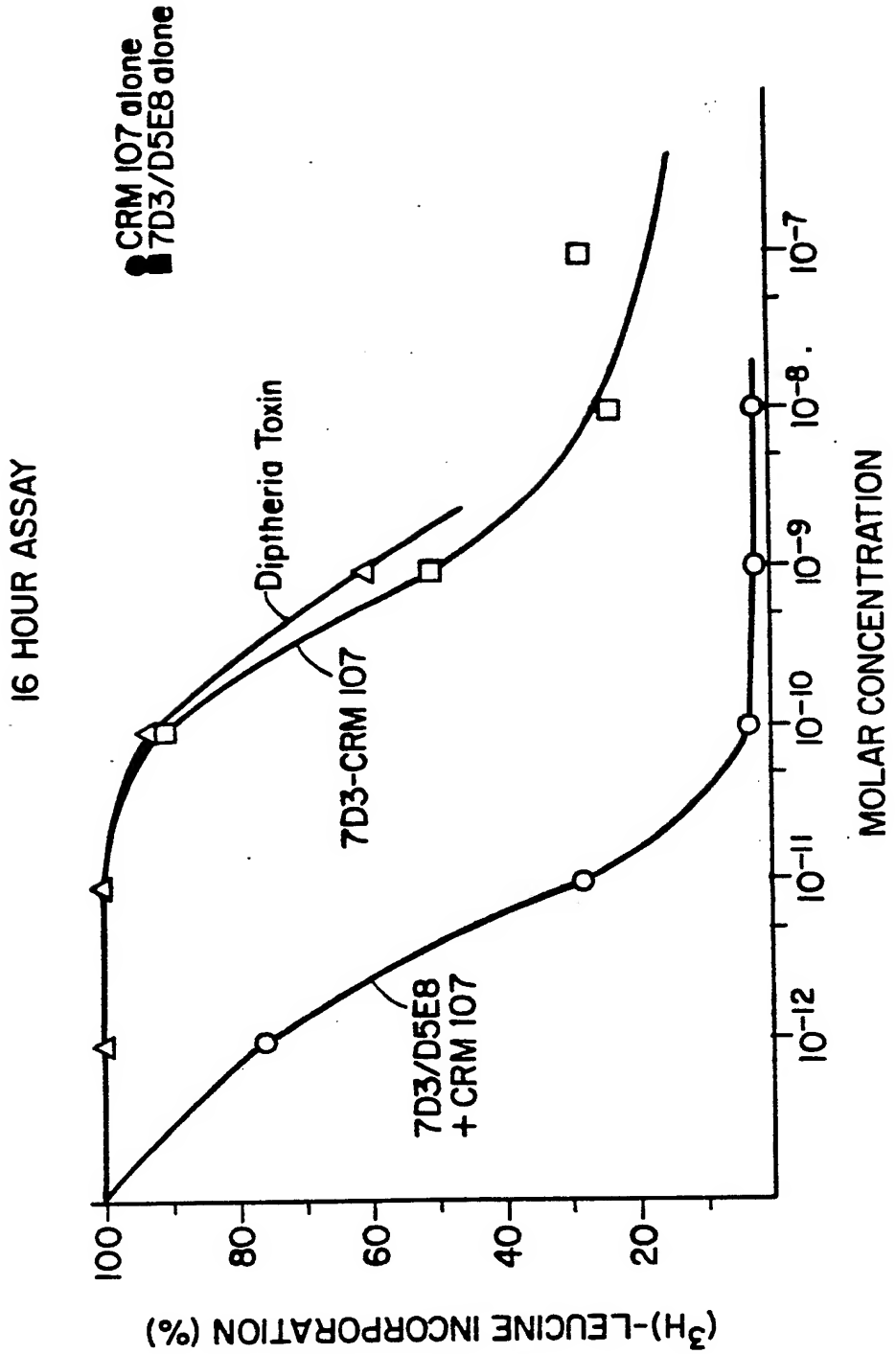
16 HOUR ASSAY
CRM 107 alone
7D3/D5E8 alone
Diptheria Toxin
7D3-CRM 107
7D3/D5E8
+ CRM 107
100
80
60
40
20
(%) ^{3}H)-LEUCINE INCORPORATION
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
MOLAR CONCENTRATION

Fig. 6

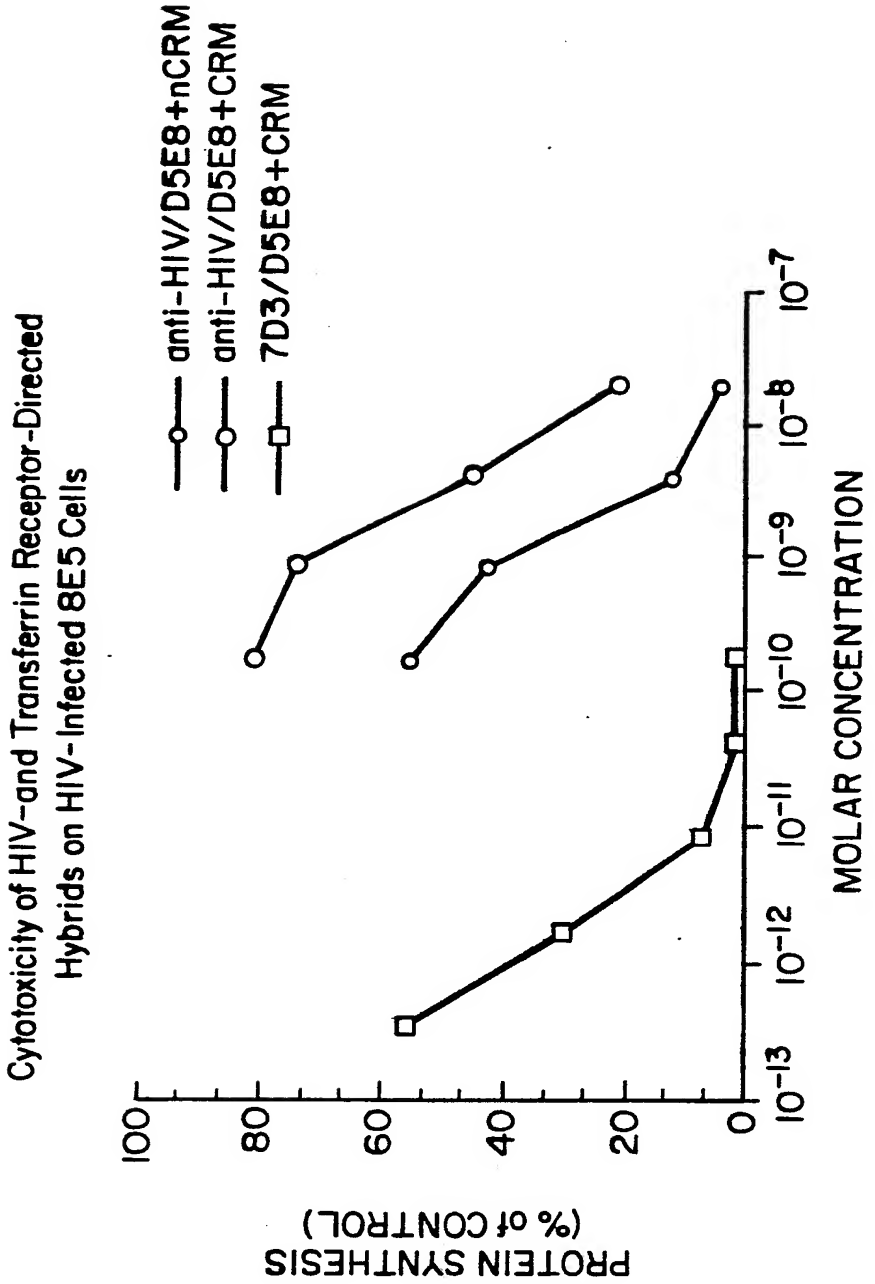
Cytotoxicity of HIV-and Transferrin Receptor-Directed Hybrids on HIV-Infected 8E5 Cells
anti-HIV/D5E8+nCRM
anti-HIV/D5E8+CRM
7D3/D5E8+CRM
PROTEIN SYNTHESIS (% of CONTROL)
100
80
60
40
20
0
10^{-13}
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
MOLAR CONCENTRATION

Fig. 7